MATERIALS AND METHODS

Cell culture, cholesterol loading and efflux. Mouse VSMC were isolated from thoracic aortas of 8-10 week-old C57BL/6 mice as described[1]. Cells were grown in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells with a passage number <5 were used in all described experiments. Peritoneal macrophages (PMφ) were harvested from C57BL/6 mice 4 days after thioglycollate injection by peritoneal lavage as described[2]. Isolated PMφ were plated on glass coverslips. After 1h incubation at 37°C, nonadherent cells were removed by gentle washing and the remaining cells were cultured overnight in DMEM containing 10% FBS before use in the phagocytosis assay.

Cholesterol loading and unloading. Cholesterol was delivered to VSMC by using Chol:MβCD complex (Sigma, catalog #C4951). The cyclodextrin (CD)-cholesterol complex was used because of the relative ineffectiveness in the past of using LDL and modified LDL-based donors to cholesterol-load VSMC *in vitro*[3]. Indeed, incubation of VSMC with 50 μg/mL of acetylated LDL for 72 h failed to increase cellular cholesterol levels or to perturb the gene expression of VSMC or macrophage-associated factors (H. Nishi, E. Fisher, data not shown). Reports more recent than reference 14, however, have described protocols *in vitro* under which VSMC can take up cholesterol from LDL and triglyceride-rich lipoproteins[4,5]. It will be interesting to include these protocols and physiologically relevant donors in future studies to determine whether any cellular effects we have observed are specific to the particular source of cholesterol that is used to load the VSMC.

Subconfluent VSMC were incubated with Chol:MβCD (10 μg/ml) in 0.2% (w/v) BSA for 72 h. Cells incubated with 0.2% BSA for 72 h without Chol:MβCD treatment served as controls[1]. For cholesterol efflux experiments, cholesterol loaded VSMC were treated with ApoA1 (100 µg/ml), HDL_3 (100 µg/ml), or β-Cyclodextrin (50 µg/ml) for 72 h. HDL_3 was isolated by sequential ultracentrifugation of human plasma; ApoA1 was obtained after delipidation of HDL_2; β-Cyclodextrin was purchased from Sigma (C4805-5G).

Transfections with micro RNA mimics or adenoviral vectors did not change the extent of cholesterol-loading (Supplemental Fig. X).

Phagocytosis and efferocytosis assays for VSMC and macrophages. Mouse aortic VSMC and PMφ plated on glass coverslips were preincubated with or without cyclodextrin cholesterol for 72 h, then incubated with 1µm Fluoresbrite™ Microsphere latex beads (Polysciences) for 24 h as described[1]. Digital images were captured by a confocal microscope using different excitation wavelengths for latex beads and cells (transmission images). To quantitatively determine the uptake of latex beads by VSMC, the numbers of beads were counted in five randomly selected fields in each experimental condition. Phagocytotic activity was expressed as the number of beads divided by the number of cells in the fields.

For the efferocytosis assay, Jurkat cells were labeled with Cell Tracker Orange CMRA and rendered apoptotic by cycloheximide treatment (100μg/mL) for 6 h. Apoptotic Jurkat cells were added to VSMC and bone marrow-derived macrophages (BMDMs) plated in a LabTek Chamber Slide system at a 5:1 ratio and incubated for 2 h. Media were removed, and cells were washed 4X with ice cold 1X PBS to remove uninternalized apoptotic cells. VSMC and macrophages were fixed and stained with Phalloidin Green and Dapi for microscopy. For efferocytosis engulfment quantification, cells having internalized apoptotic bodies were counted in 6 images/condition (10X objective, with an average of 450 cells/image) and results were expressed as % efferocytosis (# macrophages with engulfed apoptotic cells / total # macrophages x 100).

Histology and immunohistochemistry. Mouse VSMC were fixed in 4% paraformaldehyde in PBS and stained with Oil Red O (for neutral lipids) as described[6]. Immunostaining was also performed using following primary antibodies: rat anti mouse CD68 (Serotec MCA 1957S; dilution, 1:250), anti actin, α-Smooth Muscle - alkaline phosphatase (ACTA2) (Sigma A5691; 1:200), and rabbit monoclonal anti-caldesmon (CALD1) (Abcam ab32330; 1:200). After washing in PBS, cells were incubated for 20 min at room temperature with rabbit biotinylated anti-rat IgG (Vector Labs BA4001; 1:200) followed by alkaline phosphatase avidin–biotin complex (Vector Laboratories)[7] or FITC-conjugated goat anti-rabbit (Jackson ImmunoResearch Labs 111-095-144; 1:200). For CD68/CALD1 double staining, tissue sections were incubated with a mix of primary antibodies, Alexa Fluor® 488 CD68 (Serotec MCA1957A488T) and anti-CALD1 overnight at 4^{o}C followed by incubation with anti rabbit Texas Red.

Western blotting. Mouse VSMC were processed for Western blotting as described[8]. SRF was detected using the primary antibody (1:200) from Santa Cruz (sc335) and peroxidase-conjugate anti-rabbit secondary antibody (Sigma A-0545; 1:2000); quantification was performed by densitometry analysis using Quantity One software from Bio-Rad.

Quantitative Real-Time RT-PCR (qRT-PCR). Total RNA was extracted from cultured VSMC with TRIzol® (Invitrogen) according to manufacturer's protocol. cDNA synthesis was carried out with Verso™ cDNA Kit (Thermo Scientific). SYBR Green-based real time PCR (ABsolute™ Blue QPCR SYBR® Green ROX, Thermo Scientific) was used to measure mRNA or miRNA levels. Control housekeeping genes were Gapd (for mRNA) and U6 (for miRNA). The primers used for different target mRNA are listed in Supplemental Table 1. Custom miRNA assays (System Biosciences, Cat. #CSRA640A-1) were used to detect miRNA levels.

Adenoviral-mediated gene transfer. Adenoviral preparations for Ad-MYOCD and Ad-LacZ were used as described[9]. Subconfluent mouse VSMC were incubated either with Ad-MYOCD, or with Ad-LacZ (negative control) at a multiplicity of infection (moi) of 50, 100, and 300 in DMEM/2% FBS overnight at 37^{o}C. After removal of virus, transduced cells were cultured in DMEM/10% FBS for 6 h and then used for cholesterol loading experiment. A similar approach was used to for a recombinant adenovirus that expresses HA-tagged truncated nuclear SREBP-2 (Eton Bioscience Inc.), which is not

subject to proteolytic regulation and thus is in the active, nuclear, form. For either the Ad-MYOCD or Ad-SREBP-2 experiments, there was no obvious toxicity to the cells related to the treatments.

MicroRNA overexpression studies. Subconfluent mouse VSMC were transfected with 30 nM precursor microRNA mimics (Cat. #AM17100, PM12730, PM13039, Applied Biosystems) overnight using siPORT NeoFX (Applied Biosystems) following the manufacturer's instructions, then incubated with 0.2% BSA or Chol:MβCD (10 μg/ml) for 72 hours.

Microarray analysis. Total RNA was extracted from the control and cholesterol-treated mouse VSMC by using Trizol method. RNA samples were labeled and fragmented using the GeneChip 3' IVT Express kit, RNA samples were reverse transcribed to cDNA, and then hybridized onto the GeneChip® Mouse Genome 430 2.0. The complete set of microarray data that were generated in this study is available from NCBI GEO under accession number GSE47744. In addition to transcriptome profiling, we performed transcription factor binding site motif enrichment analysis and Principal Component Analysis (PCA) of mouse VSMC and hematopoietic cells.

Transcriptome profiling. Probe-level intensities were background-adjusted, quantile normalized, and summarized into probe set intensities using the Robust Multichip Average procedure[10] in Bioconductor (version 2.6). The probe-to-probe set mappings that were used in this procedure were based on Ensembl Gene identifiers (release 61) and obtained from the Custom CDF project[11] (version 14). For downstream analysis, only probe sets that had a condition- averaged $\log_2$ expression intensity of at least 6 or an intensity fold-change of at least 2.0 (up or down) between averaged intensities from the baseline and cholesterol-loaded samples were retained.

Analysis of promoter sequences. Genes were selected for promoter scanning based on a gene-by-gene statistical test of the $\log_2$ intensities between the control and cholesterol-treated sample groups (one-way ANOVA, Benjamini-Hochberg[12] false discovery rate cutoff of 0.05, corresponding to a P value cutoff of 0.0078). In addition, a minimum absolute fold-change of 3.0 was required between baseline and cholesterol sample groups. Ensembl Gene identifiers for the resulting 147 upregulated and 78 downregulated (three-fold or greater) probe sets were mapped to Ensembl transcripts (445 and 342 transcripts, respectively), and the chromosomal coordinates of the transcripts were obtained using BioMart (Ensembl release 61).

Genomic sequences ± 1 kbp from the annotated transcription start sites were obtained using the UCSC Genome Browser (mouse build NCBI37/mm9). A 3.9 Mbp "background" sequence file of promoter sequences for randomly selected transcripts associated with genes whose probe sets have $\log_2$ intensity ≥ 7 in baseline or cholesterol-loaded SMCs, was used to determine the motif-specific background probabilities of chance transcription factor binding site (TFBS) motif matches within genes that are expressed in VSMC. Two sets of promoter sequences, for cholesterol-upregulated and cholesterol-downregulated genes respectively, were scanned against a library of pre-

TFBS sequence motif patterns (TRANSFAC ver. 2010[13]) using the program Clover[14], to identify motif patterns whose matches within the promoters of cholesterol-responsive genes are more frequent than expected by chance based on the background set of promoter sequences. For the scanning using Clover, a minimum match score of 6 and a default pseudocount value of 0.1 were used. Motifs were then filtered based on the enrichment significance ($P \leq 0.01$) and whether the corresponding transcription factors were detected above background ($\log_2$ intensity ≥ 7) on the microarray in at least one condition. For identifying SRF-binding CArG elements within the promoters of up- and down-regulated genes for display in the microarray heatmap, the TRANSFAC motif V$SRF_Q6 was used (minimum Clover match score of 8). Secondary computational analysis of phylogenetically conserved CArG box element enrichment within these promoter regions was carried out using a genome-wide set of 98,236 conserved (vertebrate phastCons score > 0.971) CArG box elements mapped to the mm9 mouse genome[15]. The frequency of CArG boxes per bp within promoters of SMC3 expressed genes was computed within the background sequence file (defined above for the TRANSFAC analysis), and enrichment tests for CArG boxes within promoters of cholesterol-upregulated and cholesterol- downregulated genes were carried out using the exact binomial test.

Principal Component Analysis (PCA). PCA produces a succession of principal components (PC), each of which is a vector of scores for each gene that represents a way of linearly combining the gene expression values. The principal components are numbered in decreasing order of their power to explain variation in the gene expression measurements across all of the samples. Because each principal component has to be orthogonal to all of the other principal components, different principal components usually reflect different (substantially non-overlapping) processes or effects across the samples. In the case of Fig. 2B, principal component 1 (PC1) explains 60.5% of the variation in the gene expression measurements across the indicated cell types and samples, while principal component 2 (PC2) explains 14.0% of the variation of the gene expression measurements.

The PCA presented in Fig. 2 included microarray data from the present VSMC study, as well as from from five other studies, each based on cultures of primary mouse cells of different hematopoietic lineages and source tissues, that were obtained from NCBI GEO (accession numbers GSE12505, GSE8868, and GSE29949) and from Array Express (accession number E-TABM-310). In total, 27 microarrays were obtained from these five studies, and all were based on the Affymetrix Mouse Gene 430 2.0 GeneChip platform. Probe-level intensity data for the immune cell microarrays and the VSMC microarrays were co-processed using RMA as described above, and probe set-summarized using Ensembl Gene probe sets from the Custom CDF project (see above). Probe sets were filtered to retain only those for which the average $\log_2$ intensity in any combination of cell type and condition was ≥ 7, and for which the standard deviation of the average $\log_2$ intensity across the two VSMC conditions (untreated and cholesterol-treated) or across all immune cell conditions, was ≥ 1.0. The intensity values for the 2,967 probe sets that satisfied the minimum intensity and variability filters were mean-centered and the first five principal components (PC) were computed using the singular

value decomposition method, using the R package pcaMethods. The loading values for all 31 microarrays across all five components were extracted and used to generate the PCA scatter plot.

Statistics. Duplicate or triplicate wells were used for each treatment within an experiment, and at least two independent experimental studies were performed for each type of experiment, with the combined results expressed as average ± SD. Significance tests for mRNA levels, confocal images quantitation, and protein levels were analyzed by one-way ANOVA using PRISM software (GraphPad, San Diego). P values of < 0.05 were considered significant. Significance testing for the CArG box analysis was carried out using the exact binomial test in the R statistical package (version 2.15.1).

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